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Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties

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ABSTRACT

Four varieties of cultivated blueberries (*Vaccinium corymbosum*) and a wild crop (*Vaccinium miyrtillus*) originating form the Modena region in Italy (Mirtillo nero dell'Appennino Modenese) and protected by the mark of origin, were examined in order to determine their antioxidant activity as related to their phenolic composition. The antioxidant activity was measured as radical scavenging activity, ferric reducing activity, and by an amperometric method; the total phenolics and total anthocyanins were determined by colorimetric methods; individual anthocyanins were evaluated by HPLC. Results showed that total phenolics and total anthocyanin concentrations were, respectively two fold and three fold higher in the wild fruits, which also had a higher anthocyanin-to-total phenolic ratio. Determination of individual anthocyanins put in evidence some differences between the cultivated and wild varieties, in particular the absence of acylated anthocyanins in wild blueberries. The antioxidant activity was much higher in wild blueberries than in the cultivated ones, and it was more related to the total phenolic rather that to the anthocyanin concentration.

1. Introduction

Many research studies have demonstrated that fruits and vegetables contain various components with antioxidant activity, which are responsible for their beneficial health effects. In addition to vitamin C, vitamin E and carotenoids, polyphenols (a wide class of components including phenolic acids, catechins, flavonols and anthocyanins), have shown strong antioxidant capacity (Cao, Sofic, & Prior, 1996; Sellapan, Akoh, & Krewer, 2002; Wang & Jiao, 2000).

Blueberries contain high quantities of anthocyanins, mainly in their glycosylated forms, flavonols (such as quercetin, kaempferol and myricetin), catechins (such as (+) catechin, (−) epicatechin and their oligomeric forms), benzoic and cinnamic acids (Hakkinen, Karenlampi, Heinonen, Mykkanen, & Torronen, 1999; Kalt et al., 2001; Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001; Sellapan et al., 2002). The composition and content of phenolic compounds in blueberries vary widely, according to the cultivar, the season and the growing location, so that it is difficult to provide unique data. Published data mostly concern the cultivated varieties, referred to as highbush blueberries and belonging to *Vaccinium corymbosum* (Kalt et al., 2001; Moyer, Hummer, Finn, Frei, & Wrolstad, 2002; Prior et al., 1998), a few data concern the antioxidant composition of lowbush varieties or wild blueberries, which generally contained higher total phenolics and anthocyanin concentrations (Kalt et al., 2001; Lee, Finn, & Wrolstad, 2004a; Madhujith & Shahidi, 2004; Moyer et al., 2002).

Due to their high phenolic content, blueberries show a strong antioxidant activity (Wang, Cao, & Prior, 1996), which seems more correlated to their total phenolic content rather than to their anthocyanin concentration (Prior et al., 1998). The consumption of blueberries has been associated with a diet-induced increase in ex vivo serum antioxidant status (Kay & Holub, 2002).

In recent years blueberries, such as other small red fruits, are more available on the Italian market, both for fresh consumption and as derived products. Awareness of the health benefits correlated to blueberry consumption has also favoured the local commercialisation of wild blueberries, which spontaneously grow in mountain areas (especially in the Alps and Apennines). These berries are available as fresh products during the growing season and are also processed into jams, juices, fruit under syrup, etc.

The present study was carried out to characterise wild blueberries grown in the Appennino Modenese area and protected by the mark of origin "Mirtillo nero dell'Appennino modenese" with regard to their phenolic composition and antioxidant activity. These berries, which belong to *V. myrtillus* L., can be collected, processed and distributed under controlled conditions only by authorised organisations in the area of origin, under the control of the Camera di Commercio di Modena, which is the owner of the mark. Two lots of wild blueberries, purchased during the ripening season in the

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growing area, and four varieties of cultivated blueberries (*V. corymbosum* L.) were analysed for their general and polyphenolic composition and for their antioxidant activity. To provide reliable results, the antioxidant activity was measured by different methods, as it is known that the determination of this parameter is influenced by many variables, such as the oxidation substrate, the oxidation mechanism and the reaction medium (Baderschneider, Luthria, Waterhouse, & Winterhalter, 1999; Rice Evans, 1999).

2. Materials and methods

2.1. Blueberries

Four varieties of cultivated highbush blueberries (*V. corymbosum*, var. Bluecrop, Goldtraube, Darrow and Patriot) were purchased directly from the producer, a small farm situated at 700 m altitude on the side of Lake Maggiore (Ecodario, Portovaltravaglia, VA, Italy). Fruits were grown according to the organic farming method and were manually picked at the commercial ripening stage, from mid to end July 2006.

Two lots of wild blueberries (*V. myrtillus*, lot 1 and lot 2), belonging to the protected mark of origin "Mirtillo nero dell'Appennino Modenese", were purchased at the beginning of August 2006 from two authorised retailers in the Appennino Modenese Region (Abetone, Italy). For every type of blueberry, each lot consisted of about 2 kg. Berries were divided into approximately 100 g portions into plastic food trays and during this operation damaged and unripe berries, as well as leaves and other extraneous materials, were eliminated. Trays containing the blueberry aliquots were put into commercial plastic food bags, sealed using the provided string, and stored at −20 °C in a freezer for 48 h before analysis. This procedure was applied since we observed that fruit homogenisation gave much better results when applied to frozen material.

2.2. Analytical methods

The following determinations were carried out on fresh fruits: colour was determined on about 20 g fresh berries (before freezing) contained in a 6 cm diameter cell with black opaque walls, using a tristimulus colorimeter (Minolta CR 210, Tokyo, Japan) and values are expressed as L^*, a^* and b^* Hunter coordinates; weight of 50 berries was determined by technical balance (Gibertini TM 1600, Novate, Italy).

All other analytical determinations were carried out on a fruit homogenate obtained by crushing about 50 g of frozen berries with a food blender (Black & Decker, Hunt Valley, MD, USA).

The homogenate was used to evaluate dry matter by the AOAC (1995) method, soluble solids (determined by digital refractometer, ATAGO DBX-55), pH and titratable acidity (Lee, Durst, & Wrolstad, 2002). Polyphenolic components were extracted by the method described by Prior et al. (1998) with some modifications: ten grams of homogenate were weighed in a centrifuge tube and added with 15 mL of acetonitrile/acetic acid (96:4, vol/vol). The mixture was stirred for 1 h in the dark and centrifuged at 11,200*g* for 10 min at 15 °C. The solids were extracted two more times using 15 and 10 mL of the extraction solvent for 15 min under shaking in the dark, and centrifuged in the above-described conditions. Finally, the gathered extracts were made up to 50 mL with the extraction solvent. Each extraction was carried out in duplicate.

Total phenolics were determined on the extract by the Folin–Ciocalteau method (Singleton & Rossi, 1965) and expressed as mg gallic acid equivalents/100 g by comparison with a calibration curve built with the pure standard compound.

Total monomeric anthocyanins were determined by the differential pH method (Giusti & Wrolstad, 2001) and data are expressed as mg cyanidin-3-glucoside equivalents/100 g, using $\varepsilon = 26900$.

Individual anthocyanins were detected and quantified by HPLC following the method described by Rossi et al. (2003) using a gradient elution system and photodiode array detection (Waters, Millford, MA, USA: pump mod. 600, photodiode array detector mod. 2996 and Empower 2 data management program). The extracts were diluted with 25 mmol/L KCl buffer pH 1.0, before filtration on 0.45 μm membrane and injection in the HPLC system. Separation was performed by gradient elution (phase A, acetonitrile; phase B, 10% formic acid in water) using a C18 Spherisorb ODS 2 column (4.6 × 250 mm, Waters, Millford, MA, USA). Peaks were identified by comparison with pure standards (cyanidin-3-glucoside, malvidin-3-glucoside and peonidin-3-glucoside, Extrasynthese, Genay, France), by retention times and by spectral data, and for overlapped peaks by LC-MS.

The antioxidant activity was determined by three different methods: radical scavenging capacity, determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH*), as described by Rossi et al. (2003); ferric reducing antioxidant capacity (FRAP), as described by Aaby, Hvattum, and Skrede (2004); amperometric method as described by Buratti, Pellegrini, Brenna, and Mannino (2001). This method required a flow injection apparatus consisting of a pump (model 880 PU, Jasco, Tokyo, Japan) and an electrochemical detector (EG & G Princeton Applied Research model 400, EG & G Princeton Applied Research, Princeton, NJ) with a flow cell equipped with a double glassy carbon working electrode, an Ag/AgCl reference electrode, and a platinum counter electrode. The carrier solution was acetate buffer 0.1 mol/L, pH 4.0 and 0.050 mol/L NaCl, and a flow rate of 1 mL/min was employed. Measurements were taken at the established potentials of +0.8 V and +0.5 V; the measured current is related to the oxidation of electroactive compounds at working electrode.

All methods were calibrated with trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the antioxidant activity values were expressed as trolox equivalents (TE).

Reagents were analytical or HPLC grade. All analyses were carried out at least in triplicate.

2.3. Statistical analysis

One-Way Anova and Multiple Range Test were preformed using the Statgraphics plus 5.1 package (Graphics Software Systems, Rockville, MD, USA). Linear regression analysis was carried out by Microsoft Office Excel 2003.

3. Results and discussion

Preliminary trials were carried out to define best conditions for the extraction and analysis of blueberries. Different extraction methods were tested using the following extraction media: acetonitrile/acetic acid (96:4, vol/vol), methanol/water/formic acid (88:12:0.1, by vol), methanol/water/formic acid (70:28:2, by vol) and methanol/acetone/water/formic acid (40:40:20:0.1, by vol) (Gao & Mazza, 1994; Kalt et al., 2001). The method reported in the Section 2 gave the highest extraction yields, both in terms of total phenolics and total anthocyanins, therefore it was chosen for the study. Particular care was taken in the preparation and storage of the extracts before analysis, as it is well-known that polyphenols and the antioxidant activity can be partially lost due to oxidation and condensation reactions. Therefore extracts were prepared while avoiding exposure to light and high temperature, they were stored at −20 °C and analysed for the polyphenolic composition and antioxidant activity within the subsequent day.

Table 1
General analytical composition of cultivated (*V. corymbosum*) and wild (*V. myrtillus*) blueberries

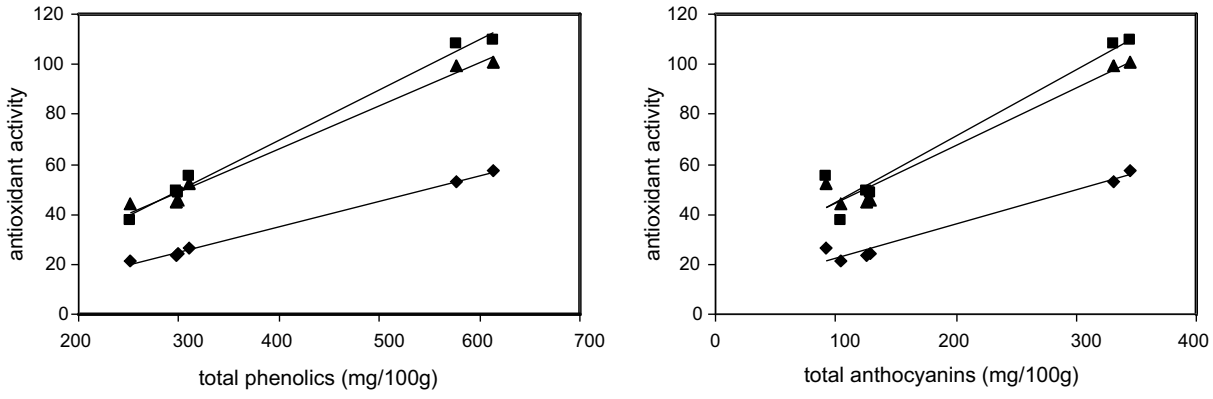
	Weight of 50 berries (g)	Total solids (g/100 g)	Soluble solids (°Brix)	pH	Titratable acidity (citric ac. g/100 g)	Colour		
						L^*	a^*	b^*
V. corymbosum								
Goldtraube	59.6 c	13.4 a	11.6 b	3.15 b	1.15 b	33.8 d	0.88 b	−2.82 ab
Patriot	79.9 e	14.4 b	12.1 c	2.92 a	1.35 c	33.9 d	2.12 c	−2.63 b
Bluecrop	39.8 b	14.6 b	12.9 e	2.90 a	1.47 d	34.9 d	0.74 ab	−3.26 a
Darrow	64.1 d	14.9 c	12.4 d	2.94 a	1.37 c	32.2 c	0.50 a	−2.47 c
V. myrtillus								
Lot 1	14.3 a	15.1 d	11.1 a	3.13 b	1.00 a	25.6 a	0.60 ab	−0.60 e
Lot 2	14.0 a	15.6 d	10.8 a	3.22 c	1.18 b	28.4 b	0.80 ab	−0.92 d
Probability level	***	***	***	***	***	***	****	***

*** $p < 0.001$. Different letters in each column indicate significant differences at 95% confidence level as obtained by the LSD test.

Table 2
Total phenolics, total anthocyanins and antioxidant activity of cultivated (*V. corymbosum*) and wild (*V. myrtillus*) blueberries

	Total phenolics (gallic ac. mg/100 g)	Total anthocyanins (cya-3-glu mg/100 g)	Antioxidant activity				
			DPPH* (TE μmol/mg)	FRAP (TE μmol/g)	EC + 0.5 V (TE μmol/g)	EC + 0.8 V (TE μmol/g)	EC ratio 0.5/0.8 V
V. corymbosum							
Goldtraube	251 a	104 b	3.79 a	21.3 a	4.42 a	11.72 a	0.377
Patriot	310 b	92 a	5.49 c	26.4 c	5.21 b	13.08 b	0.398
Bluecrop	299 b	129 c	4.86 b	24.3 b	4.57 a	12.10 ab	0.378
Darrow	298 b	126 c	4.96 b	23.7 b	4.50 a	12.03 ab	0.374
V. myrtillus							
Lot 1	577 c	330 d	10.79 d	53.2 e	9.94 c	28.46 c	0.349
Lot 2	614 d	344 e	10.96 e	57.2 f	10.08 c	29.34 c	0.344
Probability level	***	***	***	***	***	***	

*** $p < 0.001$. Different letters in each column indicate significant differences at 95% confidence level as obtained by the LSD test.



	R^2 vs total phenolics	R^2 vs total anthocyanins
DPPH*	0.9938	0.9543
FRAP	0.9975	0.9698
amperometric +0.5 V	0.9871	0. 9602

Fig. 1. Linear correlations of total phenolics and total anthocyanins versus the antioxidant activity determined by DPPH* ((■), TE μmol/10 mg), FRAP ((♦), TE μmol/g) and amperometric +0.5 V ((▲), TE μmol/10 g) methods in the various blueberry samples.

Table 1 reports general analytical data of cultivated blueberries (*V. corymbosum*, var. Bluecrop, Goldtraube, Darrow and Patriot) and wild blueberries (*V. myrtillus*, lots 1 and 2). One-way Anova shows that for all parameters, there are significant differences between samples ($p < 0.001$), and data put in evidence that wild fruits clearly differentiate from the cultivated ones.

The weight of 50 berries indicates that cultivated fruits, despite significant differences between the four varieties (Patriot has the largest fruits whereas Bluecrop has the smallest ones), have much larger berries than the wild ones. Among *V. corymbosum* varieties, general compositional data lie in quite narrow ranges: Goldtraube blueberries have a total solid content lower than the other cultivated varieties (13.4 vs. 14.4–14.9), which corresponds to the lowest soluble solid (11.6 vs. 12.15–12.9) and titratable acidity values (1.15 vs. 1.35–1.47). Consequently, the pH of Goltraube blueberries (pH 3.15) is the highest one among cultivated berries. Wild fruits show the lowest Brix value (ca 11) and the lowest titratable acidity (1.00–1.18) but also the highest total solid content (15.1–15.6); this is principally due to a higher skin-to-pulp ratio in wild berries, also related to the small berry size. Wild berries are darker than the cultivated ones, as demonstrated by the lower L^* values. Our data confirm findings by other researches on lowbush *Vaccinium* species from various origins (Lee, Finn, & Wrolstad, 2004b; Moyer et al., 2002; Prior et al., 1998).

With regard to the antioxidant composition and antioxidant activity of cultivated and wild blueberries (Table 2), data show that the total phenolic contents of cultivated berries are quite homogenous, ranging from 250 to 310 mg/100 g; total anthocyanin concentrations also lie in a narrow range (from 92 to 126 mg/100 g) and represent approximately 30–40% of the total phenolics in the four cultivated varieties. These data are in agreement with those reported by other studies for various *V. corymbosum* varieties (Lee et al., 2004a; Moyer et al., 2002; Prior et al., 1998; Sellapan et al., 2002).

Concerning the antioxidant activity, TE values show that the DPPH* radical scavenging method is more sensitive than the FRAP and amperometric methods. All values of antioxidant activity are consistent and show, among cultivated varieties, the highest activ-

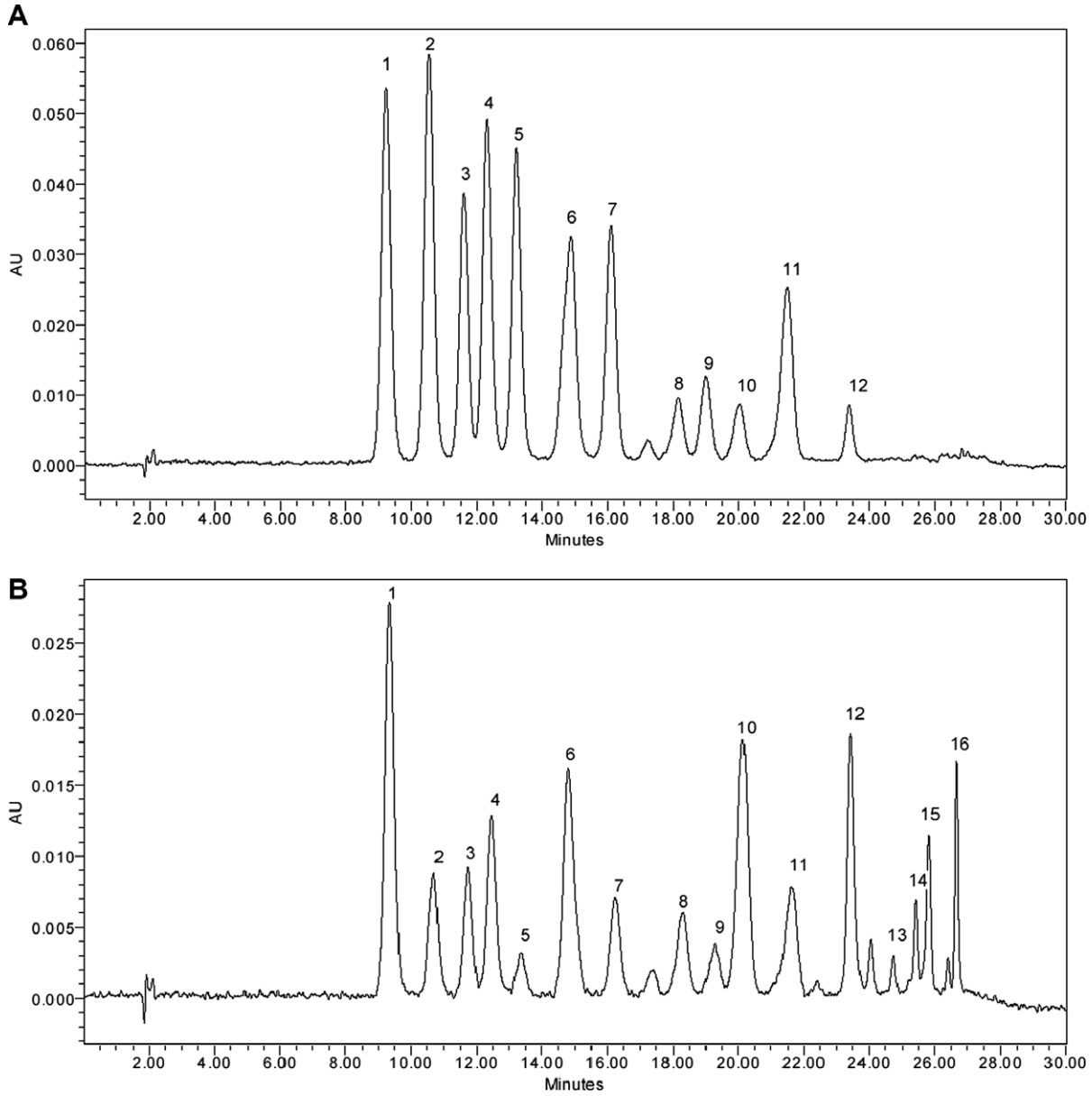


Fig. 2. HPLC profile of wild (A) and Goldtraube (B) blueberries. Peak identification: 1, del-3-gal; 2, del-3-glu; 3, cya-3-gal; 4, del-3-ara; 5, cya-3-glu; 6, pet-3-gal + cya-3-ara + pet-3-glu; 7, peo-3-gal; 8, pet-3-ara; 9, peo-3-glu; 10, mal-3-gal; 11, mal-3-glu; 12, mal-3-ara; 13-16, acylated anthocyanins.

ity in Patriot and the lowest in Goldtraube blueberries. Concerning the amperometric method, the two applied potentials were chosen considering that, as reported in previous works (Buratti, Benedetti, & Cosio, 2007; Cosio, Buratti, Mannino, & Benedetti, 2006), the antioxidant power of phenolic compounds is related to their reducing capacity, which depends on structural factors such as the number and position hydroxyl or methoxyl groups on phenolic ring. In particular, it has been demonstrated that the potential of +0.5 V is selective to discriminate those compounds having effective antioxidant power, whereas at an applied potential of +0.8 V all the phenolic compounds can be oxidised and consequently electrochemically evaluated. Linear correlation between amperometric values at +0.8 V and total phenolics, determined by the Folin–Ciocalteau method, gave a correlation coefficient $R^2 = 0.9894$, demonstrating that this potential gives good estimates of total phenolics.

Wild blueberries have much higher concentrations of total phenolics (approximately 600 mg/100 g) and anthocyanins (330–340 mg/100 g); in these fruits anthocyanins account for more than 50% of total phenolics, and are present in the pulp as well as in the skin (the pulp of wild berries is red-colored). On the contrary, the pulp of the cultivated varieties examined in this study did not contain red polyphenols. Similar results have been reported by Prior et al. (1998) on *V. myrtillus* and *V. corymbosum* samples: these authors reported concentrations of total phenolics ranging from 233 to 273 mg/100 g and total anthocyanins ranging from 62 to 157 mg/100 g in *V. corymbosum* varieties, while total phenolic content was 525 mg/100 g and anthocyanin content was 300 mg/100 g in *V. myrtillus*. Other studies investigated the polyphenolic profile of wild *Vaccinium* species as compared to cultivated ones. Lee et al. (2004a, 2004b) evaluated the polyphenolic content in various *Vaccinium* species from the Pacific Northwest of North America, finding a great variability. In particular, in wild berries total phenols varied from 489 to 702 mg/100 g and total anthocyanins from 176 to 311 mg/100 g; wild *V. ovalifolium* showed higher total phenol and anthocyanin concentrations than wild *V. membranaceum* berries. The same authors reported for cultivated *V. membranaceum* a mean content of 569 and 168 mg/100 g for total phenolics and anthocyanins, respectively; cultivated *V. ovalifolium* showed mean concentrations of 903 and 265 mg/100 g for total phenlics and anthocyanins, respectively. Moyer et al. (2002) reported for cultivated *V. membranaceum* concentrations ranging from 225 to 423 mg/100 g and from 110 to 153 mg/100 g for total phenolics and total anthocyanins, respectively.

With regard to the antioxidant activity (Table 2), wild berries show values which are more than double than the cultivated one, whatever the evaluation method, as a consequence of their higher phenolic content. Table 2 also reports EC ratio 0.5/0.8 V, which represents a "specific antioxidant activity", being obtained as the ratio between the amperometric responses at +0.5 V (antioxidant compounds) and the amperometric responses at +0.8 V (total phenolic compounds). The EC ratio is lower in *V. myrtillus* than in *V. corimbosum* varieties, indicating that the cultivated fruits have a higher ratio of highly antioxidant phenols. This result is consistent with the lower anthocyanin to total phenolic ratio in cultivated fruits, since it is known that anthocyanins have a lower antioxidant activity with respect to other phenolic components (especially flavonoids such as myricetin and quercetin, catechins and some phenolic acids) (Mannino, Brenna, Buratti, & Cosio, 1998).

Linear correlations shown in Fig. 1 demonstrate that the antioxidant activity values, determined by DPPH*, FRAP and amperometric method at +0.5 V, are slightly better correlated to total phenolics ($R^2 > 0.985$) rather than to total anthocyanins ($R^2 < 0.97$). This conclusion is in agreement with previous findings, obtained on blueberries (Prior et al., 1998) and on red wines (Giovanelli, 2005).

Table 3
Concentrations of individual anthocyanins in cultivated (*V. corymbosum*) and wild (*V. myrtillus*) blueberries

	Del-3-gal	Del-3-glu	Del-3-ara	Cya-3-gal	Cya-3-glu	Pet-3-gal + Cya-3-ara + Pet-3-glu	Peo-3-gal	Peo-3-glu	Pet-3-ara	Mal-3-gal	Mal-3-glu	Mal-3-ara	Sum acylated
V. corymbosum													
Goldtraube	454 bc	153 a	208 a	125 a	60 ab	263 a	151 a	46 ab	110 a	331 c	180 a	230 bc	309 c
Patriot	471 c	215 a	289 a	59 a	39 a	226 a	188 ab	nd	136 a	359 cd	249 a	280 c	184 b
Bluecrop	280 ab	363 a	249 a	106 a	136 b	239 a	406 c	70 b	136 a	311 bc	610 b	378 d	462 d
Darrow	184 a	209 a	142 a	78 a	95 ab	189 a	320 bc	66 b	111 a	397 d	642 b	410 d	429 d
V. myrtillus													
Lot 1	1352 d	1407 b	1197 b	634 b	716 c	769 b	921 d	350 d	178 b	243 b	831 c	187 b	nd[1]
Lot 2	1481 d	1594 b	1278 b	626 b	745 c	769 b	939 d	302 c	165 b	188 a	735 b	133 a	nd
Probability level	***	***	***	***	***	***	***	***	***	***	***	***	***

All data are expressed as μmol/kg fresh weight. Different letters in each column indicate significant differences at 95% confidence level as obtained by LSD test. *** $p < 0.001$.
[1] Not detectable.

HPLC analysis of anthocyanins allowed to identify and quantify the compounds listed in Fig. 2, which shows the HPLC anthocyanin profile of Goldtraube and wild blueberries. All analytical data of individual anthocyanins are reported in Table 2; acylated anthocyanins were not individually identified and their content is expressed as the sum of the various peaks.

The major difference in the anthocyanin profile between cultivated and wild varieties is the presence in the former of acylated anthocyanins, which are totally absent in wild blueberries. This finding agrees with data previously reported by other authors (Gao & Mazza, 1994). The four cultivated varieties show similar anthocyanin compositions, as demonstrated by the results of the LSD test (Table 3), except for del-3-gal, which was found in lower concentrations in Bluecrop and Darrow, and peo-3-gal, mal-3-glu and mal-3-ara, which were found in much higher concentrations in Bluecrop and Darrow. In general terms, delphinidin and malvidin derivatives are the most representative forms in *V. corymbosum* varieties. *V. myrtillus* showed higher concentrations in almost all the identified compounds, as expected from the higher total anthocyanin content (Table 2); this conclusion does not apply to malvidin glycosides, especially mal-3-glu and mal-3-ara, which are present in significantly lower concentrations in wild fruits than in the cultivated ones. Delphinidin glycosides are the most abundant anthocyanins in wild berries. The anthocyanin profile of *V. myrtillus* investigated in this study is similar to that reported for cultivated *V. membranaceum* from the Pacific Northwest of North America (Lee et al., 2004a).

4. Conclusions

Results obtained in this study show that wild blueberries (*V. myrtillus*) grown in the Appennine region of Italy represent a very interesting source of dietary antioxidant. With respect to some cultivated varieties (*V. corymbosum*), commonly available on the Italian market, wild blueberries have much higher antioxidant content and antioxidant activity. Total phenolic and total anthocyanin concentrations are, respectively two fold and three fold higher in the wild fruits, with a higher anthocyanin-to-total phenolic ratio in the wild fruits. The antioxidant activity of wild blueberries, determined by various methods, resulted more than double than that of the cultivated ones. The anthocyanin profiles of the varieties investigated show some differences which allow to differentiate between cultivated and wild blueberries, in particular acylated anthocyanins were detected in all the four *V. corymbosum* varieties, whereas they were absent in the wild fruits.

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